STAT3 inhibition of cholangiocarcinoma cell lines reduces viability and restricts the release of immunosuppressive cytokines in vitro.

Honors Research Thesis presented in fulfillment of the requirements for graduation with research distinction at The Ohio State University

By

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Abstract

Cholangiocarcinoma (CC) is the malignant transformation of epithelial cells originating from the bile ducts. This cancer has an abysmal 3 year survival rate of only 10% and is typically unresponsive to standard surgical and chemotherapeutic practices[7]. Therefore, novel treatment strategies are desperately needed against this malignancy. One important feature of CC cells is their autocrine secretion of interleukin-6 (IL-6)[6,9,10]. The secretion of IL-6 can activate numerous pro-oncogenic signaling pathways including STAT3 within the tumor cell, while simultaneously promoting immunologic changes in patients with advanced disease. We hypothesized that inhibition of Signal-Transducer and Activator of Transcription-3 (STAT3) pathway may elicit a dual effect by promoting apoptosis of human BC cell lines, and limiting the secretion of immunosuppressive cytokines from these cells. A panel of human CC cell lines (n=7) with various genotypic profiles demonstrated secretion of IL-6 (range 0-6593 pg/mL). Six of the seven lines assessed had constitutively phosphorylated STAT3 as determined by western blot. A novel small molecule inhibitor, FLLL100, can directly inhibit Tyr705 phosphorylation within the SH2 domain of STAT3, and induced apoptosis in the CC cell lines regardless of genotypic profile. These effects were observed within 24 hours of drug exposure at micromolar concentrations. Immunoblot analysis of PARP cleavage confirmed apoptosis. Exposure of CC cell lines to FLLL100 resulted in decreased secretion of IL-6 in culture supernatants. Together, these data indicate that the IL-6/STAT3 signaling axis plays a role in human CC survival and that targeting this pathway can limit immune suppressive factors derived from CC cell lines.

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Introduction

Cholangiocarcinoma is a malignant tumor characterized by late clinical presentation and lack of response to traditional therapeutic regimens[10]. The mortality rate from this disease is high because of its aggressive and elusive nature. The majority of patients are diagnosed with unresectable disease, and the overall five year survival rate is less than 5%[10]. There are approximately 20,000 new diagnoses annually in the United States[7]. The number of people affected globally each year is even larger[7,10]. The most frequent genetic alterations among cholangiocarcinomas are *KRAS* and *TP53*, although reported genetic alterations vary greatly[10,19]. Cholangiocarcinomas often exhibit a dependence upon the IL-6/STAT3 signal transduction pathway[6,10].

Signal Transducer and Activator of Transcription-3 (STAT3) is a transcription factor critically involved in the proliferation, survival, metastasis and immune evasion of cancer cells[1,2,4,6,21]. Activation of the STAT3 pathway can be intrinsic or extrinsic due to genetic alterations or environmental stimuli including UV radiation, chemical carcinogens or genetic alterations, respectively[3,22]. Additionally, STAT3 signaling is induced by a variety of inflammatory cytokines, including Interleukin-6 (IL-6) activation of receptor associated tyrosine kinases, often of the Janus kinase (JAK) family[3, 6]. STAT3 is also known to be activated by a variety of growth factors, including vascular endothelial growth factor (VEGF)[3, 22]. Several oncoproteins also activate STAT3, including SRC and ABL[4, 21, 22]. Upon activation, phosphorylated STAT3 molecules dimerize and translocate to the nucleus. It is possible for activated STAT3 molecules to form heterodimers with other family proteins and translocate to the nucleus[3, 4, 22]. STAT3 activity is normally rapid and transient, due to regulation by several inhibitory molecules, including suppressor of cytokine signaling (SOCS) protein[4, 6, 10].

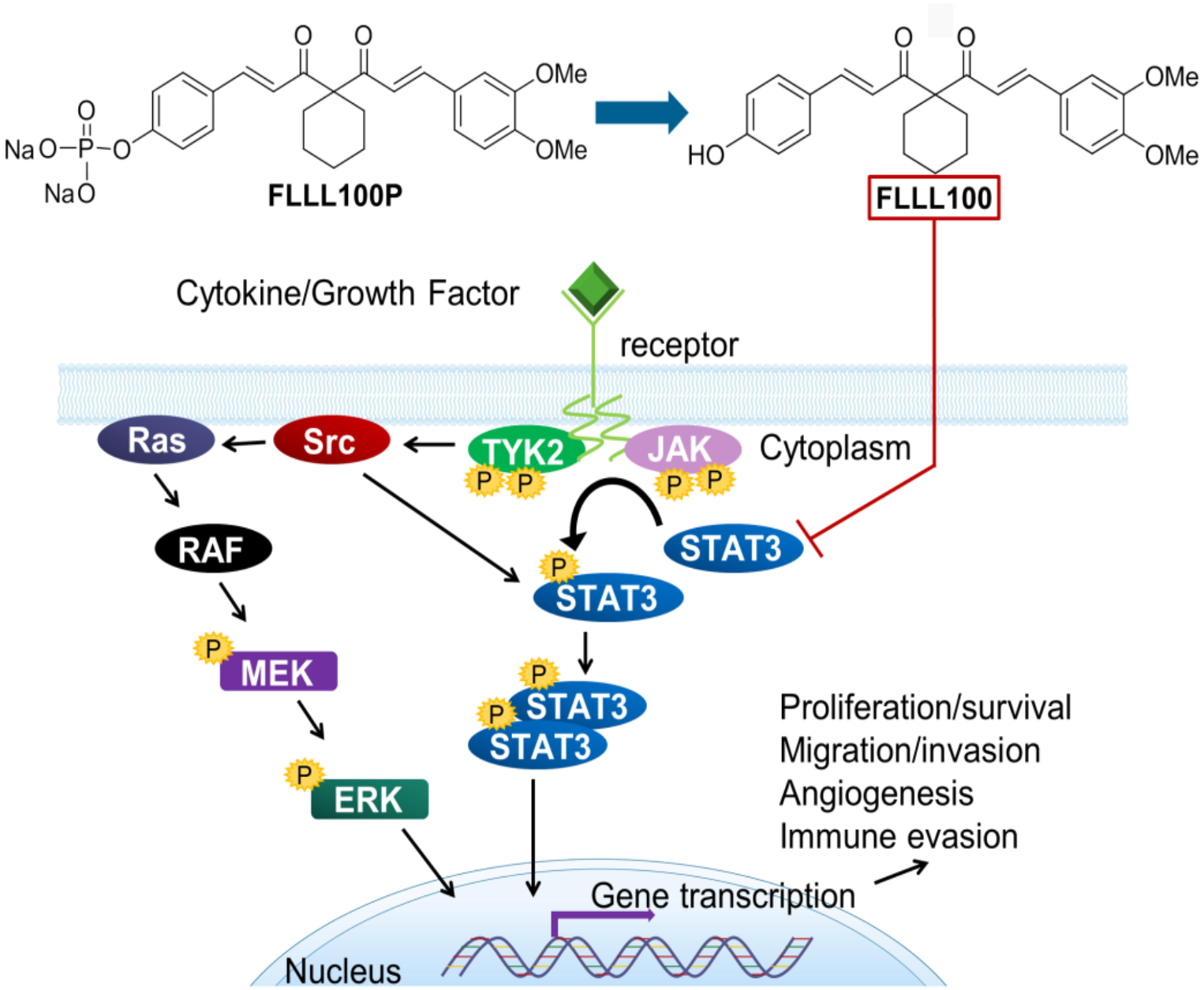


Figure 1.STAT3 Pathway. A cytokine or growth factor binds to receptor on the cell membrane. The receptor activates TYK2 or JAK. Activated kinase phosphorylates STAT3. pSTAT3 dimerizes and translocates to the nucleus. The prodrug FLLL100P is cleaved *in vivo* into the active drug FLLL100. FLLL100 inhibits the phosphorylation of STAT3.

In cancer cells, STAT3 remains constitutively activated, leading to production of gene products promoting proliferation, survival, angiogenesis and immunosuppression[6, 10, 22].

Inflammation has been implicated as a risk factor in a variety of cancers including cholangiocarcinoma. It has been previously characterized that Interleukin-6 (IL-6) is an important cytokine to cholangiocarcinoma[6, 10]. IL-6 activates the STAT3 pathway, which is known to cross-talk with other know cancer pathways including the MAPK pathway and the Ras and RAF proteins. Phosphorylated STAT3 further activates the STAT3 pathway in a positive feedback loop. As previously stated, STAT3 activation promotes proliferation, angiogenesis and survival[22], and it also promotes production of IL-6, IL-10, and other immunosuppressive factors[6,4, 22].

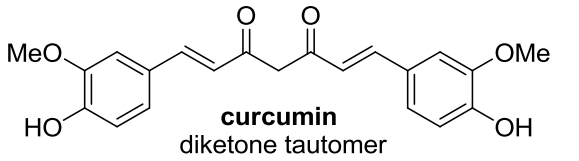
IL-6/STAT3 signaling has an immunosuppressive effect. Increased STAT3 activation promotes the generation of immature myeloid cells and increases the number of immature dendritic cells. STAT3/IL-6 signaling upregulates myeloid derived suppressor cell generation and increases generation of T- regulatory cells [17, 22]. It has been previously shown that ablation of STAT3 signaling significantly inhibits tumor growth[11, 22]. Additionally, cells transformed with STAT3-C have been characterized as tumorigenic[4]. STAT3-C is a constitutively activated STAT3 molecule made by substituting cysteine residues for specific amino acids in the SH2 domain of the STAT3 molecule[4]. We believe that STAT3 is a good target for inhibition in cholangiocarcinoma, leading to growth inhibition and apoptosis.

In the search for specific STAT3 inhibitors, some data suggest that natural products may offer a starting point for drug discovery. Previously, curcumin was of great interest in cancer research [20]. Curcumin, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione, has been shown to experimentally inhibit a variety of targets including STAT3, and exhibits chemopreventive and therapeutic properties in experimental cancer models[5]. The poor

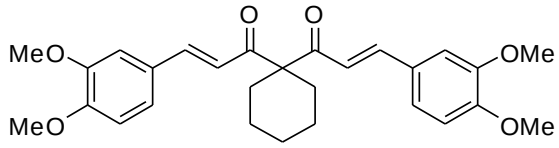
bioavailability of curcumin led to the development of analogs. These analogs, based on the diketone form of curcumin, were predicted to interact with the SH2 domain of STAT3 and prevent STAT3 homodimerization based on computational modeling[15]. As seen in Figure 2, each successive analog of curcumin has been modified slightly. Previously, our laboratory has characterized the effects of two curcumin analogs, FLLL32 and FLLL62, in melanoma and renal cell carcinoma[1, 2]. We have demonstrated that FLLL32 and FLLL62 are specific inhibitors of the JAK2-STAT3 pathway, which induces apoptosis in melanoma cell lines A375 and Hs294T[1]. Both compounds bind to the STAT3 SH2 domain, blocking homodimerization of Tyr705 phosphorylated STAT3 proteins and resulting STAT3 activation[2]. Based on these studies, a novel analog of curcumin, FLLL100, was designed in an effort to overcome the limited solubility and bioavailability of FLLL32 and FLLL62 (Figure 2). FLLL100P is a novel prodrug that has been modified with a phosphate group, which will be cleaved *in vivo* into the drug FLLL100. This modification is hypothesized to overcome the limitations in solubility seen in previous analogs. Before investigating the effects of FLLL100P *in vivo*, the effects of FLLL100 will be investigated in cholangiocarcinoma cell lines. The effects of STAT3 inhibition in human cholangiocarcinoma have not previously been characterized.

Hypothesis

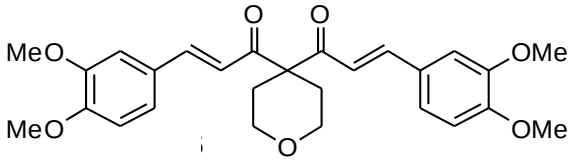
We hypothesize that inhibition of the STAT3 pathway may elicit a dual effect by promoting apoptosis of human cholangiocarcinoma cell lines, and limiting the secretion of immunomodulatory cytokines from these cells.



curcumin
diketone tautomer



FLLL32



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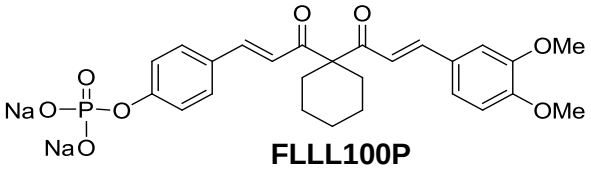
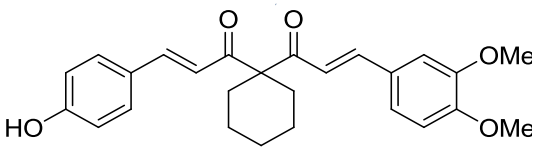


FLLL100P



FLLL100

Figure 2. Curcumin and derivatives. Poor bioavailability of curcumin led to the development of a series of analogs at The Ohio State University College of Pharmacology. The effects of FLLL32 and FLLL62 have been previously characterized in melanoma, renal, and pancreatic cancer cell lines as pro-apoptotic and anti-proliferative. FLLL32 and FLLL100 are rapidly cleared *in vivo* and FLLL100P maintains concentration in the plasma for a greater duration.

Methods

Cell Culture

Human cholangiocarcinoma cell lines HuCCT1, HuH28, WITT were a kind gift from Dr. Tushar Patel (Mayo Clinic), and MzCha1 was a kind gift from Dr. Shannon Glaser (Texas A&M Health Science Center). HuCCT1, HuH28, WITT, SNU-245, SNU-478 and SNU-1196 cells were cultured in RPMI-1640 media containing 10% FBS, 10mM L-glutamine, and antibiotics. Mz-Cha1 cells were cultured in CMRL media containing 10% FBS, 10 mM L-glutamine, and antibiotics. Cells were plated and treated with varying concentrations of FLLL100 for 24 or 48 hours. SNU-245, SNU-478, SNU-869, and SNU-1196 were purchased from the Korean Cell Line Bank (Seoul, Korea).

FLLL100

FLLL100 was synthesized in the laboratory of Dr. James Fuchs from the Ohio State University suspended in DMSO at stock concentration of 20 μM for *in vitro* studies.

Western Blot Analysis

Lysates were prepared from cell lines using Laemmli buffer and assayed from protein expression by western blot analysis with antibodies (Ab) to STAT3, pSTAT3 (Tyr^{705}), pSTAT1, pSTAT5, STAT1, STAT5, PARP, β-actin (Cell Signaling Technology) as previously described by[12]. Following incubation with the appropriate horse-radish-peroxidase-conjugated secondary Ab, immune complexes were detected using SuperSingal® West Pico Chemiluminescent Substrate (Thermo Scientific).

Cellular Growth Assay

Cholangiocarcinoma cells were seeded in 96-well plates (HuCCT1 3x103 cells/well, HuH28 $3x10^3$ cells/well, WITT $3x10^3$ cells/well, MzCha1 $3x10^3$ cells/well, SNU 245 $6x10^3$

cells/well, SNU 478 $2x10^3$ cells/well, SNU 1196 $4x10^3$ cells/well) in triplicate and allowed to adhere overnight. Fresh media containing FLLL100 was added to each well at various concentrations (Range = 0 - 10 μM) and cells were incubated at 37ºC/5% CO2 for 72 hours. Cells treated with DMSO (vehicle) served as controls. At this time, the percentage of cell growth was evaluated using the MTS assay (CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega) and quantified by determining the optical density at 595 nm using a Bio-Rad iMark™ microplate reader.

Analysis of Apoptosis via Flow Cytometry

Cholangiocarcinoma cells were seeded in 6-well plates (HuCCT1 $1x10^6$ cells/well, HuH28 $1x10^6$ cells/well, WITT $1x10^6$ cells/well, MzCha1 $1x10^6$ cells/well, SNU 245 $1x10^5$ cells/well, SNU 478 $1x10^5$ cells/well, SNU 1196 $2x10^5$ cells/well) and allowed to adhere overnight. Fresh media containing FLLL100 was added to each well at various concentrations and cells were incubated at 37ºC/5% CO2 for 72 hours. Cells treated with DMSO (vehicle) and untreated cells served as controls. At this time, phosphatidyl serine exposure was assessed in tumor cells by flow cytometry using APC-Annexin V and propidium iodide (PI; BD Pharmingen, San Diego, CA) as previously described [1, 13, 14]. Each analysis was performed utilizing at least 10,000 events on a FACSCalibur flow cytometer.

Analysis of cytokines in CC culture supernatants

Supernatants from CC cultures were assessed for the presence of IL-6 using commercial ELISA (R & D Systems, Inc., Minneapolis, MN). Fresh media containing FLLL100 was added at various concentrations to CC cells and incubated at 37ºC/5% CO2 for 24 hours. Supernatants were collected at this time. Samples were run in duplicate per manufacturer's recommendations.

Results

Majority of Cholangiocarcinoma cell lines display constitutively activated STAT3.

The STAT proteins are highly homologous and act to transduce signals across the cell membrane and as transcription factors at the DNA level[4, 21, 22]. STAT3 and STAT5 play a major role in cancer inflammation, while STAT1 plays a role in anti-tumor immune responses[22]. These pathways can be antagonizing, and represent an area of interest in targeted therapeutic interventions. The basal expression of STAT family proteins was assessed in a panel of human cholangiocarcinoma cell lines to determine if cholangiocarcinomas would be susceptible to FLLL100. In the majority of cell lines (n=6 of 7) detectable basal pSTAT3 levels were observed. Only the SNU-245 cell line lacked basal STAT3 phosphorylation. The amount of basal pSTAT3 varied among the cell lines, as seen in Figure 3. Basal levels of pSTAT5 were observed in n=3 cell lines. In contrast, no cell lines had detectable phosphorylation of STAT1, consistent with its role as growth inhibitory transcription factor[22].

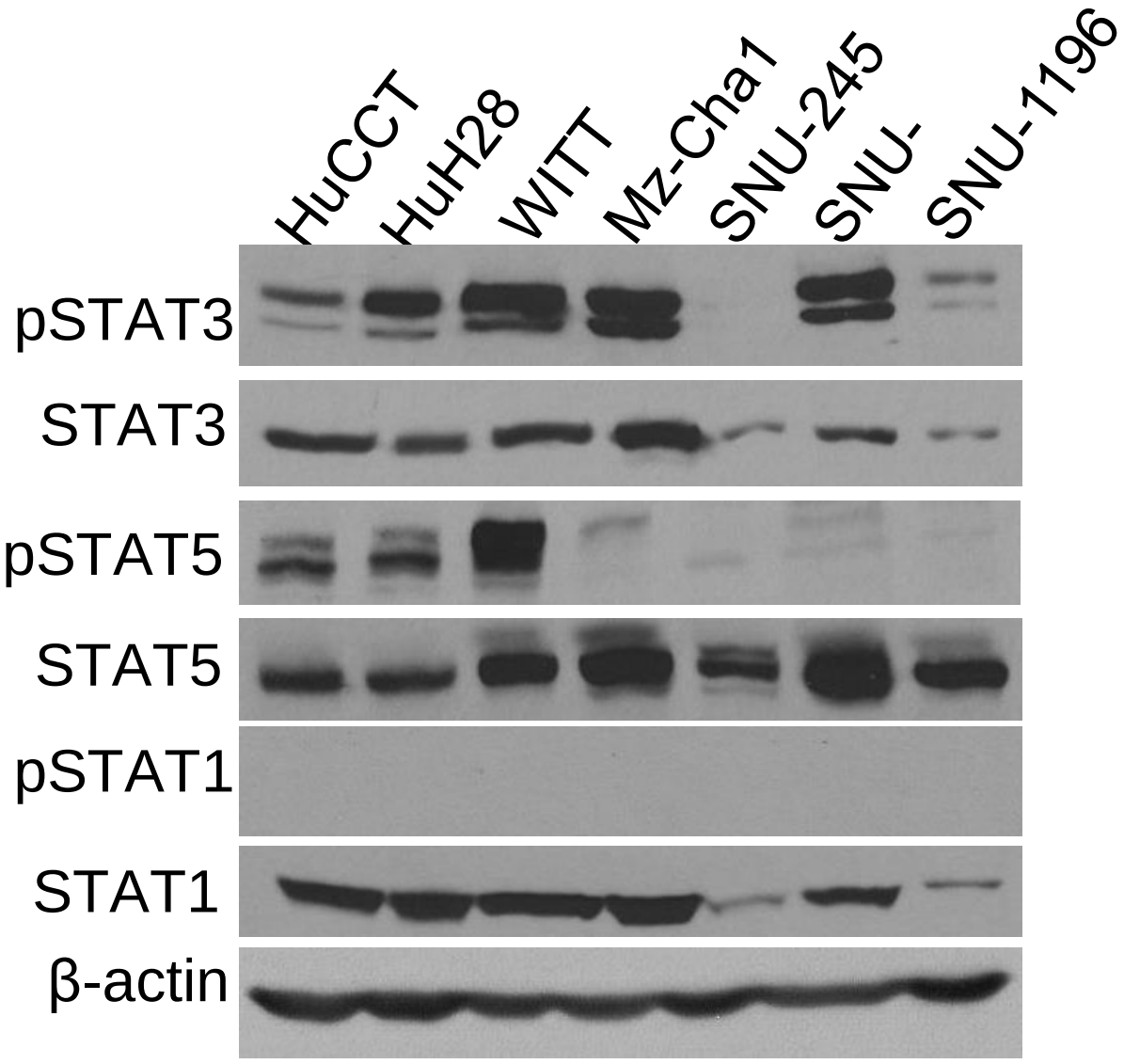


Figure 3. Basal levels of protein expression in the human cholangiocarcinoma cell lines, n=7**.** In n=6 cell lines, there was some amount of basal expression of pSTAT3. There was pSTAT5 expression in n=3 cell lines. In 7 cell lines, there was no detectable expression of pSTAT1. β-Actin was used as a loading control.

STAT3 phosphorylation decreased with FLLL100 treatment.

After observing constitutively active STAT3 in the majority of human cholangiocarcinoma cell lines, we next determined what effect FLLL100 treatment would have on pSTAT3 expression. SNU-478 was selected as representative cell line for this experiment, as it had a high level of basal pSTAT3 expression. With increasing concentrations of FLLL100, there was a decrease in pSTAT3 (Figure 4). Importantly, there was no change in the level of total STAT3 protein expression. These data indicate that FLLL100 affected phosphorylation events rather than transcription or translation of total STAT3 protein. This experiment was replicated in additional cell lines and similar effects were observed (data not shown).

FLLL100 exhibits growth inhibition in human CC cell lines.

We next tested whether FLLL100 could elicit growth inhibitory properties against human cholangiocarcinoma cell lines *in vitro*. We hypothesized that FLLL100 would exhibit potent growth inhibitory activity against human cholangiocarcinoma cell lines. In pSTAT3 positive cell lines, FLLL100 exhibited growth inhibition to different degrees. The general trend observed was a concentration-dependent decrease in percent growth in response to FLLL100 (Figure 5). In the SNU-245 cell line, there was no basal pSTAT3 (see figure 3). There was little change in the percentage of growth with increasing concentrations of FLLL100 in the SNU-245 cell line. These data provide additional support to the mechanism of action of FLLL100.

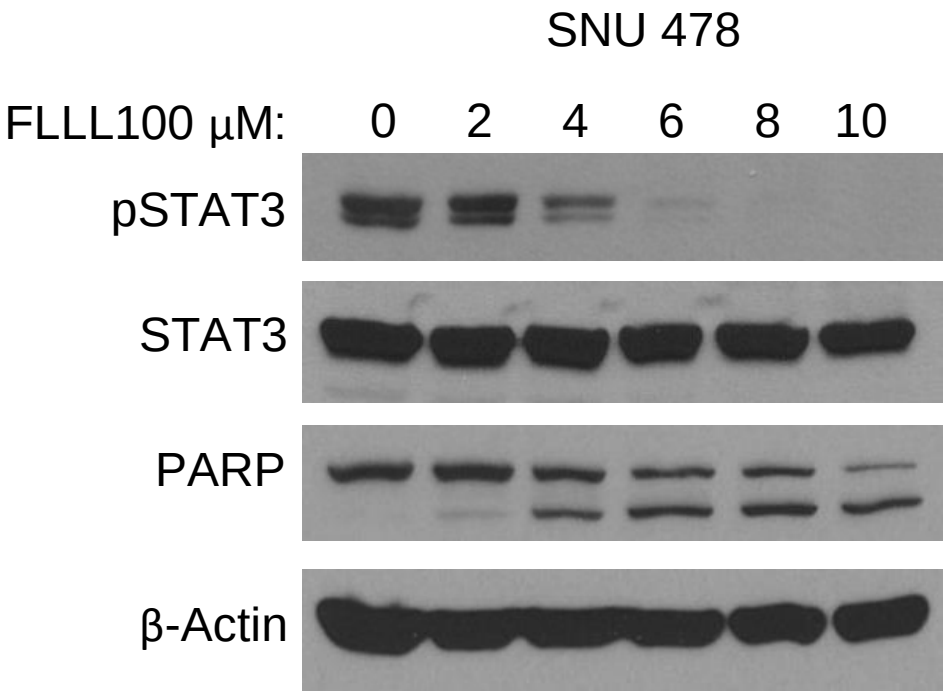


Figure 4. STAT3 expression following treatment with FLLL100. With increasing concentrations of FLLL100, there is decreased intensity of pSTAT3 observed. Additionally, there is increased PARP cleavage at higher concentrations of FLLL100. β-Actin was used as a loading control.

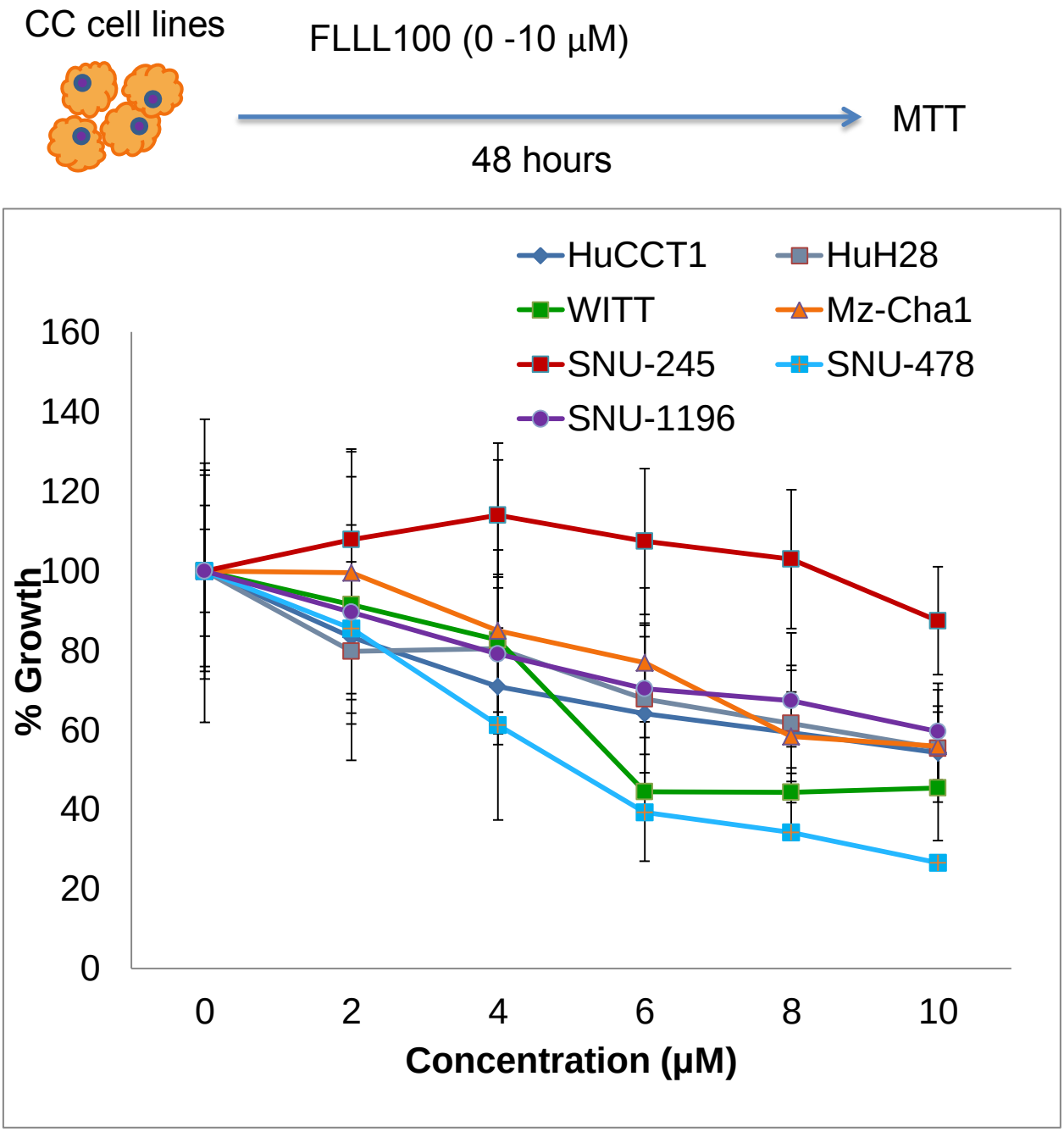


Figure 5. FLLL100 induces growth inhibition. Cells were treated for 48 hours with increasing concentrations of FLLL100. At least 2 biological replicates were performed. With increasing concentrations of FLLL100, there is a decrease in growth in n=6 cell lines.

FLLL100 induces apoptosis in human CC cell lines.

After observing the growth inhibitory effect of FLLL100, we investigated whether this agent would also promote pro-apoptotic effects. A concentration-dependent increase in apoptosis of human CC cell lines was observed following a 48 hour treatment with FLLL100 in n=6 of 7 cell lines (Figure 6). There was no observable effect of FLLL100 on apoptosis in the SNU-245 cell line, which lacked basal phosphorylation of STAT3. Again, this supports the notion that FLLL100 elicited pro-apoptotic activity by interrupting the phosphorylation of STAT3.

IL-6 production is decreased with FLLL100 treatment.

IL-6 is both an upstream activator and a gene product of the STAT3 pathway[10, 16, 21, 22]. As a result, we tested whether FLLL100 would alter IL-6 secretion from human CC cell lines. Many of the human CC cell lines secreted basal levels of IL-6, but the initial concentration observed varied greatly between individual cell lines. For example, the HuCCT1 and HuH28 CC cell lines secreted the largest basal concentrations of IL-6. In contrast, SNU-245 CC cell line secreted no detectable concentration of IL-6 (data not shown). Following treatment with FLLL100, the concentrations of IL-6 were decreased (Figure 7). This effect on reduced IL-6 was observed even at concentrations that were insufficient at inducing apoptosis at this time point.

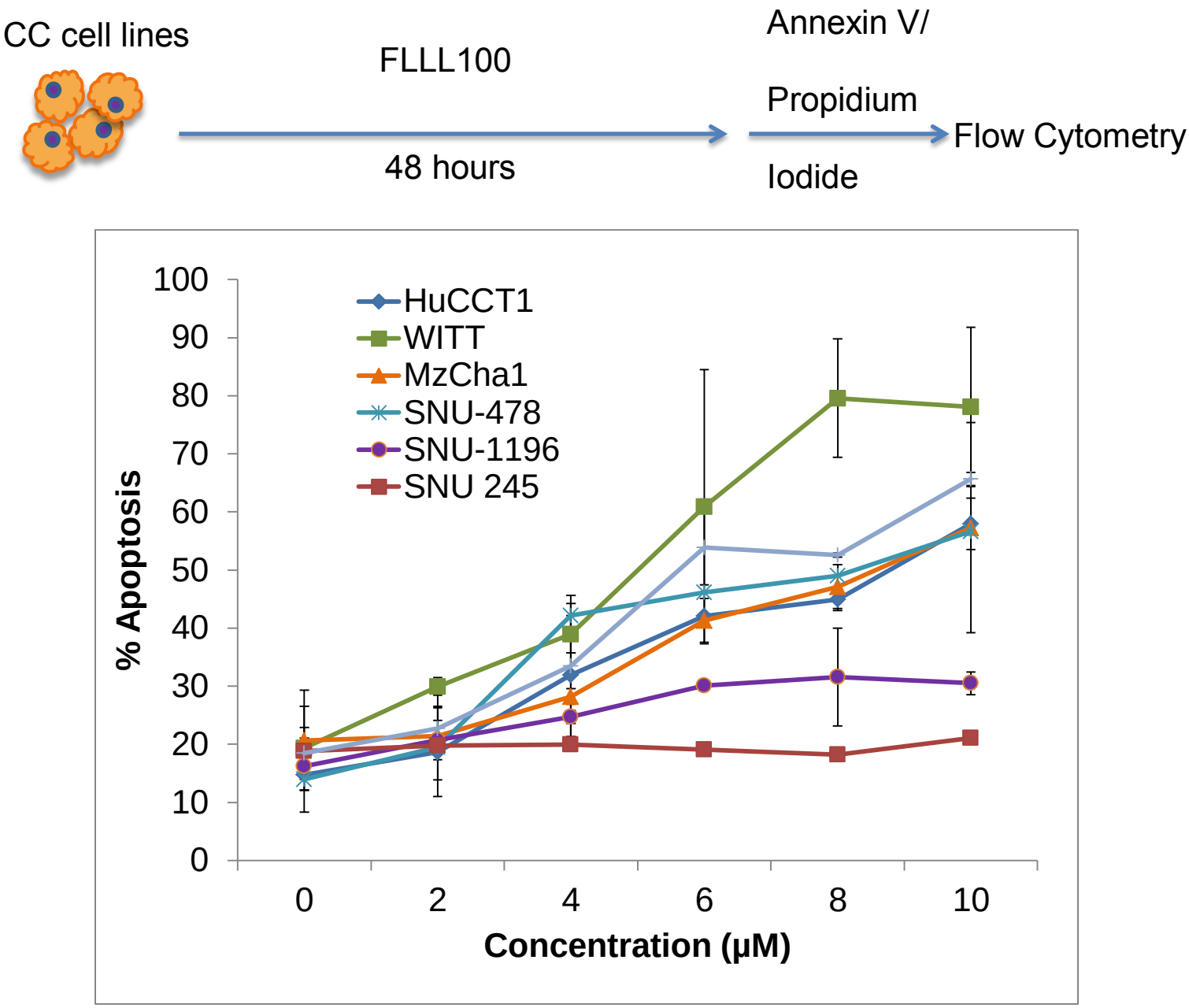


Figure 6. FLLL100 induces apoptosis in cholangiocarcinoma cell lines. Cells were treated for 48 hours with increasing concentrations of FLLL100. At least 2 biological replicates were performed. With increasing concentrations of FLLL100, there is an increase in apoptosis in n=6 cell lines.

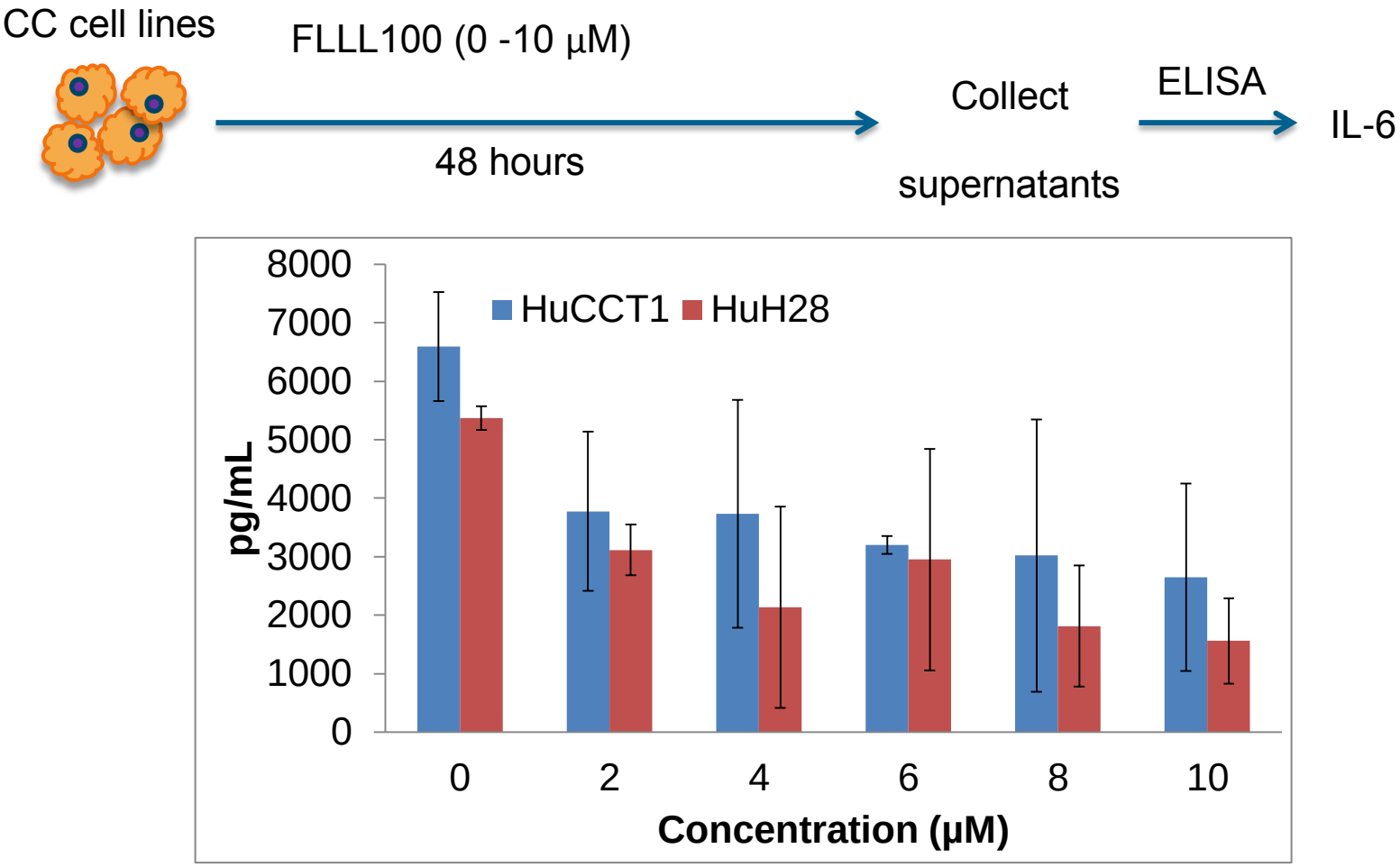


Figure 7. Decreased IL-6 production following FLLL100 treatment. Cell supernatants were collected and IL-6 concentration was assessed. At least two biological replicates were performed. With increasing concentration of FLLL100, there is decreased concentration of IL-6 observed.

Discussion

We observed that STAT3 inhibition produced anti-tumor effects using a novel small molecule inhibitor FLLL100. We showed that most human cholangiocarcinoma cell lines have basal levels of pSTAT3 present. In the panel shown, only SNU-245 is pSTAT3 negative. We demonstrated that FLLL100 inhibits phosphorylation of STAT3 in human cholangiocarcinoma cell lines, as seen in representative cell line SNU-478. In SNU-478, the level of pSTAT3 showed a decrease with increasing FLLL100 concentrations. Since SNU-245 is pSTAT3 negative, we do not expect FLLL100 to have an effect on it. We also showed that FLLL100 induced cytostatic and cytotoxic effects. We demonstrated that treatment with FLLL100 inhibits growth and induces apoptosis in human cholangiocarcinoma cell lines. We observed that FLLL100 can decrease autocrine IL-6 production in human cholangiocarcinoma cell lines. It is possible that the decreased production of IL-6 could be due in part to the decreased number of viable cells. However, there is a decrease in IL-6 observed at very low concentrations of FLLL100 (2,4 μM) as compared to the growth inhibition and apoptosis data, which did not show a decrease or increase respectively until higher concentrations of FLLL100 (6, 8, 10uM). This suggests that the decrease observed in IL-6 production is real and as a result of FLLL100 treatment, and not a side effect of the growth inhibitory or apoptotic effects of FLLL100.

We have shown that FLLL100 is novel small molecule inhibitor specific to STAT3. SNU-245 is a pSTAT3 negative cell line, which served as a useful point of comparison in many of the experiments. We expected treatment with FLLL100 to have no effect on SNU-245, because FLLL100 acts to inhibit phosphorylation of STAT3 by competitively binding at the Tyrosine 705 phosphorylation site. There was no change to the percent of growth or apoptosis respectively of SNU-245 following FLLL100 treatment. Since it appears that FLLL100 has no effect in the absence of pSTAT3, and pSTAT3 is only transiently activated in normal cells, we hypothesize that FLLL100 will have no effect on human biliary cells and will specifically target only cancer

cells. If FLLL100 also induces apoptosis in human biliary cells, then it will have limited clinical potential. However, our laboratory has previously shown that other curcumin analogs have no effect on normal human cells such as melanocytes[1,2], and we are hopeful that FLLL100 will not affect biliary cells, or any normal human cells.

We observed that inhibition of the STAT3 pathway elicited a dual effect by promoting apoptosis of human cholangiocarcinoma cell lines, and limiting the secretion of immunomodulatory cytokines from these cells. STAT3 inhibition has not previously been investigated in cholangiocarcinoma, and these results support the pursuit of further investigation. As previously described, cholangiocarcinoma patients are in desperate need for novel therapeutic options. We believe that STAT3 inhibition could be a viable treatment alternative or supplement to patients who are unresponsive to traditional chemotherapy. Indeed prior studies with other STAT3 inhibitors such as Stattic have shown the ability to sensitize cells to chemotherapy[18].

There are several limitations of this project in its current form. First, we have not yet completed studies in which normal, non-cancerous cells are treated with FLLL100 as a control. This is important because this inhibitor will have no therapeutic application if it is not specific to malignant cells. In order for FLLL100 to continue development as a cancer therapy, we must demonstrate that it has no effect on normal cells. However, using earlier structural analogs of FLLL100, our laboratory has shown that FLLL32 and FLLL62 had no effect on normal melanocytes or peripheral blood mononuclear cells from healthy adult donors[1, 2]. Based on these data, we are hopeful that FLLL100 will also have no adverse effects on normal cholangiocytes. Second, we have not yet tested this compound in more clinically-relevant mouse models of cholangiocarcinoma, which were recently acquired by our laboratory[8]. We are currently studying the best dosing regimen of the prodrug of FLLL100 (FLLL100P) using pharmacokinetics. As a result, we have not yet conducted any *in vivo* experiments. In the future,

we plan to use both these models and an immunosuppressed mouse model where human CC cells are injected subcutaneously into the mouse. Although the immunosuppressed mouse model would allow us to investigate the potential action of FLLL100P on human CC cells, it would limit us in data output. This model would give us no information about the effects of FLLL100P on the immune system, which plays an important role in the progression of cancer. To be an accurate representation of cholangiocarcinoma in patients, it would be better to use a mouse model that spontaneously develops cholangiocarcinoma as a result of genetic mutations or an extrinsic factor, which we are planning to do. Third, our experiments did not conclusively demonstrate the mechanism of action of FLLL100. We hypothesize that FLLL100 inhibits phosphorylation by competitively binding to STAT3 at the tyrosine 705 phosphorylation site. While we have a variety of data to support this, we did not pursue any experiments to explicitly show it, so it is possible that it is acting on a different protein and triggering the effects we observed. One experiment we could use to decisively show the mechanism of action would be to transfect human cholangiocarcinoma cells with STAT3-C, a constitutively activated STAT3 molecule[4]. In these cells, we would hypothesize that FLLL100 treatment would have no effect on proliferation or survival since the phosphorylation site would no longer exist.

There are still many different experiments to be conducted before FLLL100 would reach the patient level. One very essential experiment to conduct would be to investigate the effects of FLLL100 on normal cholangiocytes. Before this treatment would be approved for human trials, it would be necessary to demonstrate that FLLL100 would have no effect on normal cells. In addition to evaluating the effects of FLLL100 in normal cholangiocytes, there are many directions to take this project in the future. For example, this project could go progress to *in vivo* experiments. We have not previously investigated FLLL100 in mice, but our collaborators are working on determining the dosing in mice. We hypothesize that the prodrug, FLLL100P, will be activated *in vivo* and show improved solubility as compared to previous analogs. Additionally,

combination experiments could be performed using FLLL100 and either MEK inhibitors or JAK inhibitors. As seen in figure 1, these pathways are interconnected, and we believe that combination treatments could be even more effective.

Conclusions

FLLL100 inhibited STAT3 phosphorylation in human cholangiocarcinoma cell lines. Additionally, FLLL100 induced cytostatic and cytotoxic effects. Finally, FLLL100 decreased autocrine IL-6 production in human CC cell lines. Future experiments to assess *in vivo* efficacy of FLLL100 should be conducted. We believe that STAT3 represents a relevant target in human cholangiocarcinoma. Cholangiocarcinoma is a rare but brutal disease and there is need for novel treatments for these patients as their disease is often diagnosed late and nonresponsive to traditional treatments. The majority of cholangiocarcinomas exhibit activated STAT3, as do many other cancers, and as such, we believe FLLL100 may hold potential for future drug development as a STAT3 inhibitor.

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